DEVELOPMENTAL MASSAGE THERAPY (DMT) DURING EARLY POSTNATAL LIFE: EFFECT ON BONE GROWTH, MINERALIZATION, AND STRENGTH IN JUVENILE AND YOUNG ADULT RATS

by

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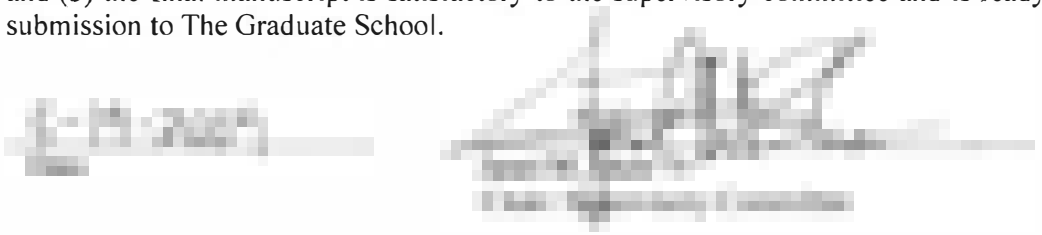

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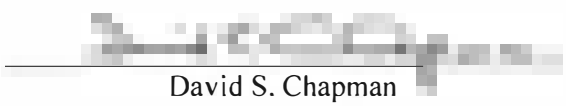

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ABSTRACT

The purpose of the current study was to investigate the effects of developmental massage therapy (DMT) during early postnatal life on growth, body composition, and skeletal development in juvenile and young adult rats. Twenty-four rat pups received a daily session of DMT (10 minutes/day) from D6 to D10 of postnatal life and were compared to matched controls (CTL, n=24). Body composition, soft tissue lean mass, fat mass, bone mineral density (BMD), bone mineral content (BMC), and bone area (BA) were measured by peripheral dual energy X-ray absorptiometry (pDXA); bone strength (Peak Load) and intrinsic stiffness on femur shaft were tested by three-point bending (MTS); cortical and cancellous bone histomorphometric measurements were performed on the tibia shaft and metaphysis. Food and water intake were monitored and did not differ among groups between weaning (D21) and D60. Body weight, body length and soft tissue lean mass at D21 were significantly greater in the DMT cohort; at D60 soft tissue lean mass was still greater in DMT groups but no differences were detected in body weight and body length. Moderate to strong correlations were found between body weight and soft tissue lean mass and measured bone outcome variables. BMD by DXA was significantly higher in D21 DMT rats and BA was only greater in D60 DMT males. Femur diameter and length were significantly greater in D60 DMT males. Bone strength and the endosteal percent mineral surface were significantly increased in D60 DMT females compared to CTL. In addition, DMT treatment improved the cancellous bone mineral surface, mineral apposition rate and bone formation rate on the trabecular bone

surface area in D21 female rats. Treatment and sex interactions were found for BMC and BA at D60. In summary, DMT during early postnatal life elicited anabolic effects on muscle-bone relationships in juvenile (D21) and young adult (D60) rats. These findings were confirmed histologically by increased percent mineral surface and mineral apposition rates. It is speculated that the positive skeletal outcomes were, in part, due to a modulation of stress by DMT resulting in an improved balance of the neuroendocrine system and regulation of the muscle-bone unit. The absence of biochemical markers as well as body composition and bone studies in older animals suggest a need for future studies to verify whether early life DMT has a prolonged positive impact on lifelong skeletal health.

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INTRODUCTION

Review of the Literature

Infants and Perinatal Stress

Stress experienced during the perinatal period, which encompasses both in-utero and early postnatal life, may contribute to the development of adult disease (Barker et al., 1989, 2000; Boloker et al., 2002; Simmons et al., 2001). This Barker Hypothesis raises concern about possible deleterious consequences of environmental stress during the perinatal period.

A newborn infant's ability to respond to physical and environmental stressors is dependent upon the balance between: 1) the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS), which are the two branches of the autonomic nervous system (ANS) and 2) the endocrine system, specifically the hypothalamic-pituitary-adrenal (HPA) axis (Ellis et al., 2006). In this dynamic relationship, the SNS activates the "flight or fight" response leading to increased release of cortisol by the HPA axis while the PNS promotes 'rest and restoration' via pituitary gland stimulated growth

hormone (GH) release and hepatic tissue generated insulin-like growth factor (IGF) with resultant somatic growth. Previous research has shown that the PNS develops later in gestation (~37-38 weeks), and young infants (< 36 weeks) tend to have an overriding SNS which leads to physiological instability and sustained stress response (Brosnan, 2001). Thus, a greater balance between the ANS/HPA axis systems leads to better physiologic stability (Kapoor et al., 2006) and should improve postnatal neurodevelopment outcomes for infants. Previous research has shown that the PNS develops later in gestation (~37-38 weeks) and young infants (< 36 weeks) tend to have an overriding SNS which leads to physiological instability and sustained stress response (Brosnan, 2001).

Massage Therapy in Infants

Gentle tactile stimulation or 'touch' and infant massage therapy have been promoted as an effective complementary intervention for stress reduction and enhancement of postnatal growth and development in term and prematurely born infants (<37 weeks gestation) (Underdown et al., 2006; Vickers et al., 2004). Massage therapy focuses on the skeletal and soft tissues of the body and uses hand-applied force and movement to general or specific areas of the body with the goal of assisting the body in self-regulation and healing. The type of massage therapy generally utilized in infants is a gentle, slow stroking of each part of the body. Some techniques also included kinesthetic stimulation consisting of passive flexion and extension of the limbs or vestibular or auditory stimulation.

Physicians have been reluctant to provide supplemental stimulation to preterm infants because the stimulation associated with handling and invasive procedures may lead to adverse physiologic reactions in newborn infants (Field, 1980). Recent studies of noninvasive forms of stimulation such as massage, however, have generally reported improvements in growth and/or behavioral development (Field, 1980; Ottenbacher et al., 1987). Supplemental tactile/kinesthetic stimulation studies performed in preterm infants (Field et al., 1986; Scafidi et al., 1990) have shown enhanced growth, less stress behavior, and better performance on developmental assessments at 8 months of age (Field, 2002, 2006; Hernandez-Reif et al., 2007; Scafidi et al., 1993). Kuhn et al. (1991) found that a tactile/kinesthetic stimulation paradigm that enhanced postnatal weight gain and neural behavior in preterm infants also facilitated a normal developmental rise in catecholamine excretion. Specifically, integrated sampling under nonstressful conditions demonstrates a highly stable individual pattern of catecholamine and cortisol activity during early human development. Similar findings have been reported in term newborns (Underdown et al., 2006). Thus tactile/kinesthetic stimulation appears to promote ANS/HPA axis maturation and tone in newborn infants during early postnatal life. The proposed neuroendocrine mechanisms of infant massage therapy are shown in Figure 1.

Systematic reviews of massage or gentle still 'touch' therapy studies in premature, low-birth-weight (< 2, 500g), or term infants from the 1960s to present, however, identified only 23 randomized clinical trials among more than 100 publications. The most common technique incorporated massage and kinesthetic stimulation for 10-15 minutes,

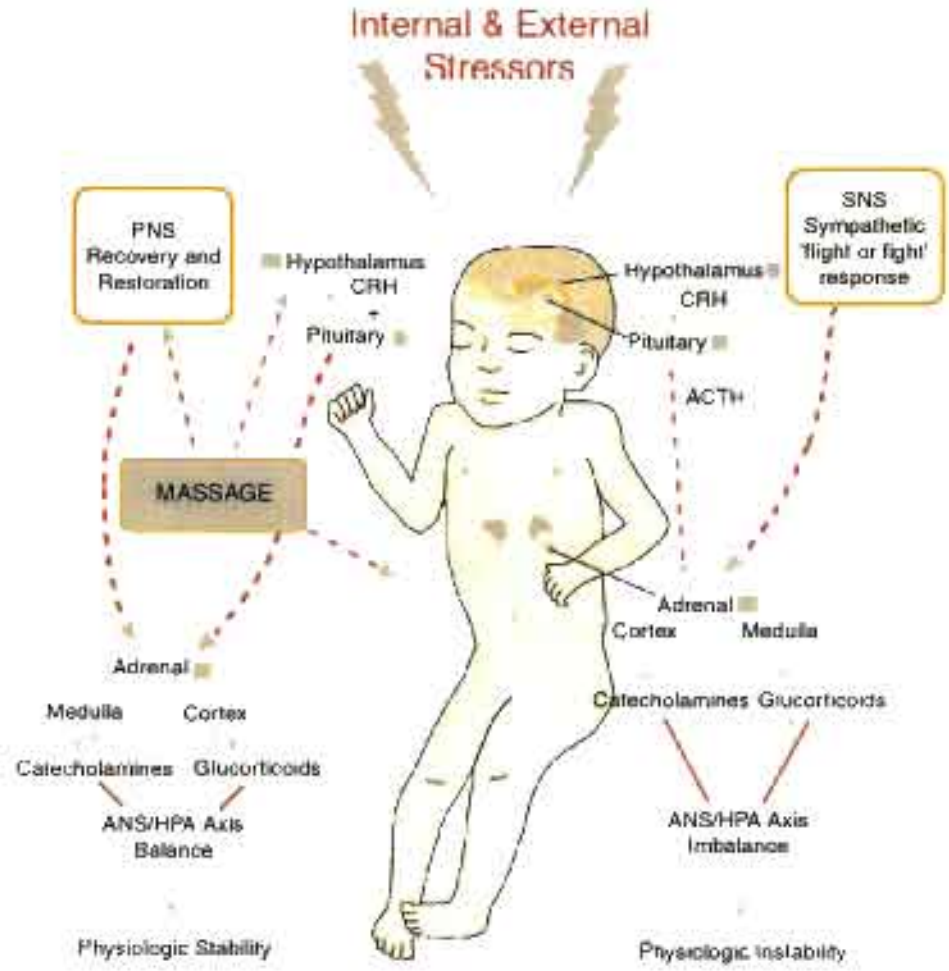

Figure 1. Proposed neuroendocrine mechanisms of infant massage therapy (Moyer-Mileur et al., 2007).

2-3 times daily for up to 10 days. Although clinical significance could not be determined, massage intervention in preterm infants appeared to improve daily postnatal weight gain by 5.1g/day and decrease the length of hospital stay by 4.5 days. 'Touch' or tactile therapy alone did not impact either weight gain or length of stay. Infants born at term gestation (>37 weeks) who received massage therapy during the first 6 months of life were approximately 300g heavier than term infants who received routine maternal care (Underdown et al., 2006). Behavioral and developmental outcomes in both preterm and

term infants were similar when compared to controls. Several serious concerns regarding the methodological quality of the reviewed studies were identified and to date, they have limited the enthusiasm for the integration of massage therapy into routine care for premature or term infants.

Postnatal Skeletal Growth in Infants and Children

Among determinants of osteoporotic fracture, there is a growing body of evidence to suggest that poor growth during intrauterine and early postnatal life might be associated with an increased likelihood of both low bone mass (Gale et al., 2001) and fracture (Cooper et al., 2001) in later adulthood. Premature infants or infants who experienced poor growth in utero are at risk of developing osteopenia and rickets because of 1) limited accretion of bone mass in utero, 2) a greater need for bone nutrients than in infants delivered at term, and 3) decreased postnatal calcium retention (Koo et al., 1989, 1991, 1995; Rigo et al., 1998). Although provision of bone-related nutrients at advised levels increases bone mineralization, postnatal mineralization rates are less than in-utero mineralization rates despite normal body weight gain (Koo et al., 1989; Tsang et al., 1993). Bone mineralization in preterm or growth-restricted infants does not approach normal ranges until after the 1st year of life (Abrams et al., 1989) and may continue to be inadequate into childhood, increasing the risk of future fracture (Helin et al., 1985).

Postnatal Skeletal Growth, Mineral Deposition, and DMT

The most effective way to prevent osteoporosis may be to increase bone mineral content (BMC) during childhood, thereby developing a stronger skeletal foundation to offset age-related bone loss (Fassler & Bonjour, 1995; Slemenda et al., 1994). Mechanical loading on bones and joints stimulates bone formation and growth (Yeh et al., 1993). Osteoblasts, the cells responsible for bone formation, increase activity in response to mechanical loading in vitro (Schultheis, 1991). Mechanical loading or weight-bearing activity increases bone mass in children, young adults, and older individuals (Davee et al., 1990; Pirnay et al., 1987; Pocock et al., 1986). Absence of mechanical loading, as seen in spaceflight and bedridden adults, increases bone resorption and hypercalcuria decreases bone mass (Mazess & Whedon, 1983). Standard care of hospitalized, preterm infants includes swaddling or nesting and decreased sensory and physical stimulation (Moyer-Mileur et al., 1995, 2000). Thus, hospitalized, preterm infants experience limited movements, which may contribute to increased bone resorption and demineralization.

Moyer-Mileur et al. (1995, 2000) have previously shown a positive effect on radius bone mineralization in preterm, very low birth weight (VLBW <1, 500 g) infants who received daily touch and kinesthetic movements similar to those used in "infant massage therapy" studies. More recently these authors compared preterm infants who were randomized to receive daily physical activity administered by their mother (MOM), a trained therapist (OT), or control. The intervention consisted of range of motion movements against passive resistance to all extremities for 5 to 10 minutes daily. Dual energy x-ray absorptiometry (DXA) of the forearm bone area (BA, cm^2), mineral content (BMC, g) and density (BMD, g/cm^2), and biochemical measures of bone formation and

resorption were obtained at study entry and at 2.0 kg of body weight. Greater forearm BA and BMC gains were found in MOM and OT infants compared to the control infants despite similar postnatal growth rate and nutrient intake. Serum bone formation levels decreased in controls but remained unchanged in MOM and OT infants. Thus greater bone growth and mineral acquisition was independent of who administered the daily intervention, i.e., the infant's mother or a trained therapist. Further, the similarities between the intervention used by Moyer-Mileur et al. (1995, 2000, 2008) and others (Litmanovitz et al., 2003, 2004, 2007; Nemet et al., 2002) and earlier infant massage studies (Field, 2005), however, suggest that either improved ANS/HPA axis balance, mechanical strain and loading, or a combination of these effects may improve postnatal bone growth and mineral acquisition in premature infants. Similar studies have not been conducted in growth-restricted infants. Therefore, additional studies are needed to elucidate the mechanisms of developmental massage therapy (DMT, touch with kinesthetic movements) on postnatal ANS/HPA axis balance and skeletal growth and bone health. To answer these questions animal models of DMT are needed to gain a better understanding of the impact of DMT mechanisms that regulate bone tissue growth and development during early postnatal and later life.

Animal Models of Touch Therapy

In animal models, maternal separation produces lasting changes in physiology and behavior (Anand & Scalzol, 2000). For example, maternal separation from postnatal day 9 (D9) to D18 disrupts maturation of the adrenal stress response (Heinrichs & Koob, 2001). More recently, it has been reported that maternal separation disrupted adult rat

leaning, long-term potentiation, and hippocampal synaptic organization (Brunson et al., 2005).

In the early 50s, Weininger (1954) first demonstrated that neonatal "gentling," i.e., the stroking of newborn pups for 10 minutes very day for the first three weeks of life, was able to attenuate later "physiological damage" and behavioral fearfulness in albino rats. This early experience effect became more intriguing when Levine (1957) and Levine and Lewis (1959) demonstrated that simply separating the mother and pups daily for as little as 3 minutes had similar effects. This treatment called "early handling" (EH, or neonatal handling, postnatal handling) appeared to attenuate the deleterious effects of severe stressors.

Several variants of the direct action hypothesis were proposed attributing the EH effects to cooling of the pups while separated from the dam (Schaefer et al., 1962) or stress imposed on the pups by the treatment (Levine et al., 1956). However, others proposed that EH might act indirectly on the pups via its effects on the nature of maternal behavior (Barnett & Burn, 1967; Bell et al., 1974; Denenberg et al., 1964; Richards, 1966; Smotherman et al., 1977 a, 1977 b).

Maternal care, in rats, occurs in the form of nursing bouts, with the dam approaching the pups and gathering them under her body while licking them and eventually assuming an upright, crouching posture, in which [the dam] stands over all or most of the pups with a pronounced dorsal arch (Stern & Johnson, 1990). The temporal distribution of nursing bouts, their frequency and duration, and possibly some qualitative aspects such as the dam's nursing posture, seem to be mediated by a complex interplay of both pup cues and maternal motivation (Meaney, 2001; Pryce & Feldon, 2003;

Smotherman & Bell, 1980; Stern et al., 1990). Despite supporting evidence that 1) prohibiting interaction between mother and pups following EH significantly alters the effects of EH (Villescas et al., 1977), and 2) EH has long lasting effects on mother-infant interactions (Lee & Williams, 1975, 1977), it was not until the late 1990s that the maternal mediation hypothesis was systemically tested (Caldji et al. 1998; Francis & Meaney, 1999; Liu et al., 1997). These authors examined changes in maternal behavior induced by EH, and whether individual variation in EH-induced changes in maternal behavior would correlate with stress and fear responses of nonhandled offspring when adult. Indeed, EH was found to stimulate active maternal care as expressed by increased levels of licking and grooming and arched-back nursing (as opposed to more passive nursing postures), and that levels of licking/grooming and arched-back nursing in dams of nonhandled pups negatively correlated with measures of stress and fear in their adult offspring (Caldji et al., 1998; Francis & Meaney., 1999; Liu et al., 1997; Meaney, 2001). Based on this paradigm, Meaney and colleagues further demonstrated that individual differences in stress reactivity induced by maternal programming are transmitted across generations (Francis & Meaney, 1999), and may be mediated by epigenetic changes in gene expression (Weaver et al., 2004).

Postnatal Stress and Skeletal Growth in Rat Pups

As previously discussed, stress such as maternal or postnatal undernutrition during early life may permanently change body structure, physiology and metabolism and leads to chronic diseases in later life. Undernutrition during different critical time periods around birth in rodent models has been documented to have long-lasting effects on body

composition and skeletal growth, Engelbregt et al. (2004) examined body weight and body composition in pubertal rats and adult rats of 6 months after pre- and postnatal malnutrition. Three treatments were tested: prenatal malnutrition, intrauterine growth retardation (IUGR) induced by uterine artery ligation, and postnatal food restriction (FR) by litter enlargement to 20 pups per mother from D2 to D24 (weaning). Both IUGR and FR resulted in persistent growth retardation with body weight and total body bone mineral content were significantly lower in IUGR until 6 months. Chahoud and Paumgartten (2005) investigated the relationship between term fetal rat (E21) body weight at term and the extent of ossification of fore- and hindlimbs and cervical and coccygeal vertebrae. Evaluation of the skeleton and the extent of ossification of fore- and hindlimb phalanges and of cervical and sacrococcygeal vertebrae was dependent on fetal body weight. The strongest correlation between body weight and degree of ossification was found for hindlimb, medial and proximal phalanges. Their data suggest that, in full-term rat fetuses, the reduced ossification of sternum, metacarpus, and metatarsus were due to a localized impairment of bone calcification (i.e., a malformation or variation) rather than from general growth retardation. Further this study suggested that ossification of hindlimb (media and proximal) phalanges is a good indicator of treatment-induced fetal growth retardation.

A similar rat model of IUGR was induced by uterine artery ligation at day 18 of gestation (Lane et al., 2001, 2002, 2003). The Lane model was employed to compare postnatal bone growth, mineralization, and bone strength in IUGR female rats during early postnatal life and adulthood (Moyer-Mileur & Chen et al., 2008). IUGR femur and tibia were compared to controls at D0, D7, D21, and D120. All animals were housed in

similar conditions, weaned at D21, and received ad lib feedings of standard rat chow from D21 to D120. IUGR body weight and femur/tibia lengths were significantly lower at all time points. Bone growth assessed by measurement of hypertrophic cell length and the primary ossification center, and size-adjusted bone strength were similar between IUGR and controls at D0, D7, and D21. At D21 size-adjusted whole body BA BMC, and BMD values were also similar. At D120, however, IUGR had lower size-adjusted BA and BMC and decreased hindlimb bone strength. These findings demonstrate a long-term negative effect of an early life stressor (IUGR) on subsequent adult bone size, mineral content, and strength.

Early Handling or Massage Therapy in Rat Pups

EH appears to mediate alterations in maternal behavior, particularly increased licking and grooming of the pups (Levine, 1994). The effect of EH on behavioral and neuroendocrine responses to stress represents one of the classical models for the study of environmental regulation of neural development and function. In adult animals, EH decreases behavioral and endocrine response to stress (Meaney et al., 1996). During development, EH rat pups opened their eyes earlier, developed motor coordination faster, and gained more body weight than nonhandled pups.

Rodent models provide strong evidence of the potential for massage therapy to modulate the HPA/ANS axis response to stress. In animals, postnatal stress such as maternal separation, repetitive pain, or undernutrition in early life may permanently change body structure, physiology, and metabolism increasing the incidence of adult chronic diseases. Maternal separation during early postnatal life disrupts maturation of

the adrenal stress response (Kuhn & Schanberg, 1998), alters cortisol (GC) expression, and negatively impacts adult learning, long-term potentiation, and hippocampal synaptic organization (Meaney et al., 1996). Similar to humans, undernutrition during critical time periods around birth in rat pups has long-lasting effects on body composition and skeletal growth. "Massage-like" tactile stimulation during early postnatal life improves ANS balance and tone (Chatterjee et al., 2007; Holst et al., 2005; Kurosawa et al., 1995; Lund et al., 2002), pain control (Alasmi et al., 1997), and weight gain (Lund et al., 2002) and decreases behavioral and endocrine response to stress in adult animals (Van Oser et al., 1998). Most recently, Chatterjee et al. (2007) elegantly demonstrated that daily "massage-like" stimulation during early postnatal life reversed altered neural protein expression elicited by extreme maternal isolation. The hypothesized effects of postnatal stress and DMT on stress and postnatal growth and bone formation are presented in Figure 2.

Sex-specific Differences in Postnatal Growth

Under normal conditions rat dams spend more time caring for their male rather than for their female offspring (Moore & Morelli, 1979), which could imply that the effects of EH may be sex-dependent. Sex steroids also participate in the control of energy homeostasis and postnatal growth and body tissue deposition. Androgens are "anabolic" agents that increase food intake and lean mass, whereas estrogens are "catabolic" and decrease food intake and body weight in rats (Mystkowski & Schwartz, 2000). Androgen deficiency induced by orchiectomy in male rats leads to a 10-15% permanent reduction in food intake that is reversed by testosterone replacement (Gentry & Wade, 1976).

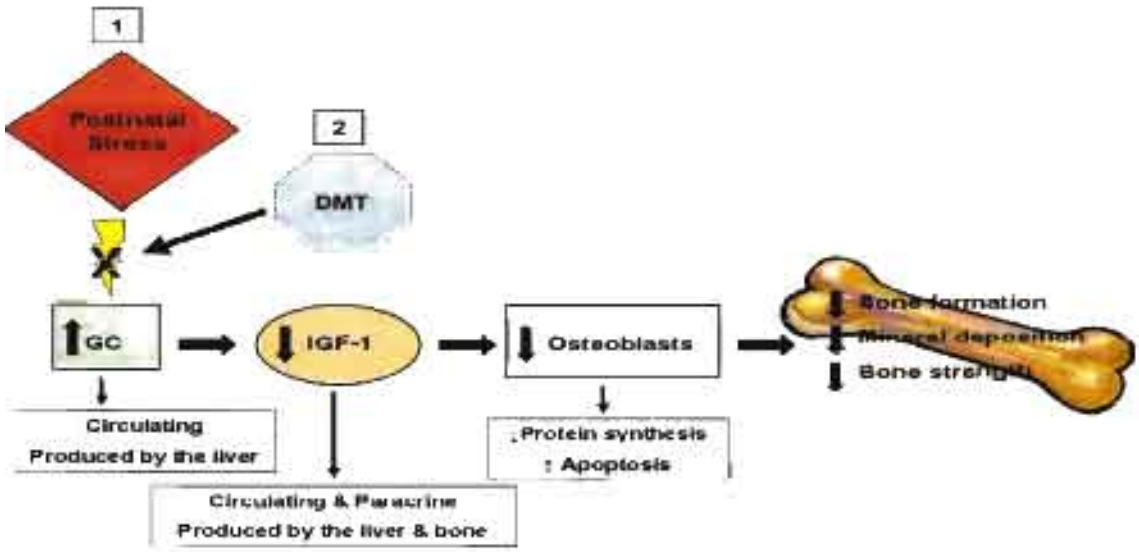

Figure 2. Hypothesized effects of postnatal stress and DMT on GC, IGF-1, osteoblast activity, bone formation, mineral deposition, and strength (NIH-NCCAM-R21AT004185-01; Moyer-Mileur & Smith, 2007).

Physiologic estrogen levels are negatively correlated with food intake in female rats during estrus (Blaustein & Wade, 1977) and, in rats fed on normal chow, ovariectomy leads to an increase in food intake that is reversed by physiologic replacement of estrogen (Asarian & Geary, 2002; Wade, 1975). Both estrogen and leptin reduce food intake but only estrogen has been associated with illness or malaise-related taste aversions (Bernstein et al., 1986).

Sex differences in bone size, architecture, and formation rates have also been documented in young rats. Compared with female rats, male rats have 11.6% longer tibiae, a 27.8% greater cortical bone area, and a 37.6% greater trabecular separation. Conversely, female rats have greater cortical (316%) and cancellous (145%) bone formation rates, 28.6% more cancellous bone, and 30% greater trabecular number

(Hefferen et al., 2003). Trabecular thickness, however, does not differ by sex. The architectural changes in cancellous bone were associated with decreases in bone formation and steady-state mRNA levels for bone matrix proteins and cancellous bone resorption (Hefferen et al., 2003). Therefore, significant sex-related differences in bone mass and turnover are present in young rats.

It is possible that the observed differences between male and female rats are related to skeletal maturity and gonadal status rather than to sex. Histomorphometric examination of the proximal tibial metaphysis reveals that female rats have a higher cancellous bone volume (normalized to tissue volume) than male rats. The more abundant cancellous bone mass in female rats can be attributed to estrogen. Secretion of estrogen at puberty reduces resorption of calcified growth plate cartilage during endochondral ossification (Budy et al., 1952; Lindquist et al., 1960) by inhibiting the fusion of chondroclasts from their circulating precursors (Turner et al., 1994a). The volume of primary spongiosa is thus increased without an increase in longitudinal bone growth (Turner et al., 1994a). Increased retention of primary spongiosa provides a more extensive template for deposition of bone matrix by differentiating osteoblasts, leading to an increase in cancellous bone volume without an accompanying increase in bone formation rate (BFR) (Turner et al., 1994a). The unanticipated sex difference in the periosteal BFR, where female rats had a higher BFR but lower cortical bone mass, may be related to the inhibitory effect of caloric restriction on male rats. Underweight male rats ate less food and lost more weight than comparable female rats. Caloric restriction has been shown to be a potent inhibitor of radial bone growth (Turner et al., 1991). The greater cancellous bone volume in female rats is associated with differences in bone

architecture. Trabeculae were more closely spaced than in male rats, but there was no sex difference in trabecular thickness. A higher bone formation rate in female rats is due to an increase in mineralizing surface as there was no difference in mineral apposition rate. This bone loss was accompanied by architectural changes; there were decreases in trabecular number with corresponding increases in trabecular space.

Summary

Early life events, especially perturbations of the mother-infant relationship, appear to alter homeostatic imbalance in the offspring and increase behavioral and physiologic-related pathological disorders in adulthood. The effects of certain environmental stimuli may persist throughout the life of the human or animal especially if such stimuli occurred during a time of maximal sensitivity or "critical period". Several examples of environmental regulation of neuroendocrine development in both infant and rat models have been presented. Massage therapy appears to attenuate negative stimuli received by both infants and rat pups in early life. Although improved body weight, bone growth and bone mineralization in premature infants in response to massage therapy have been reported, our knowledge on how massage therapy impacts bone during early postnatal life, puberty, or adulthood in a tissue- and/or sex-specific manner is limited. Therefore, an animal model for massage therapy in early life is needed to better understand the molecular mechanisms underlying the physiological changes seen in preterm and term infants.

Significance of the Study

Little is known about how massage therapy during early postnatal life modulates skeletal growth and development at the tissue level or whether differences persist into childhood, adolescence, and adulthood. An animal model provides the opportunity to determine how massage therapy during early postnatal life influences bone tissue and subsequently bone health in later life. Once bone tissue responses are described, future studies at the molecular level can be designed that will help identify massage mechanisms in bone tissue and refine therapeutic interventions for vulnerable infant populations.

Statement of the Problem

The impact of massage therapy during early postnatal life on bone tissue growth and development has not been addressed in a neonatal animal model. Specifically, it is important to determine how massage therapy during early postnatal life affects postnatal growth and patterns of skeletal growth, bone mineralization, and bone strength.

Research Questions

This study was designed to investigate the following research questions:

1. Did developmental massage therapy (DMT) administered to rat pups during early postnatal life influence bone growth, mineral deposition, and strength at weaning (D21)?

2. Did DMT stimulation administered to rat pups during early postnatal life influence bone growth, mineral deposition, and strength at puberty (D60)?
3. Did DMT stimulation during early neonatal stress influence bone growth, mineral deposition, and strength in a sex-specific manner?

Hypotheses

The following hypotheses were tested in this study:

1. Body size, bone growth, mineral deposition, and strength are greater at weaning (D21) in rat pups randomized to DMT versus Control (CTL) during early postnatal life (D6-10).
    1) Body size indices (body weight and body length) and percent lean body mass measured by DXA are greater in DMT than CTL at D21;
    2) Bone growth indices (bone formation rate by fluorescence labels in tibia shaft and proximal metaphysis) would be greater in DMT than CTL at D21;
    3) BMC and BMD measured by DXA are greater in DMT than CTL at D21;
    4) Bone strength index (bone microarchitecture by histomorphometry, and Peak Load by three-point bending) are greater in DMT than CTL at D21.
2. Lean body mass, bone growth, mineral deposition, and strength are greater in pubertal rats randomized to DMT versus CTL during early postnatal life (D6-10).
    1) Body size indices (body weight and body length) and percent lean body mass measured by DXA would be greater in DMT than CTL at D60;

2) Bone growth indices (bone formation rate by fluorescence labels in tibia shaft and proximal metaphysis) are greater in DMT CTL at D60;
3) BMC and BMD measured by DXA are greater in DMT than CTL at D60;
4) Bone strength index (bone microarchitecture by histomorphometry, and Peak Load by three-point bending) are greater in DMT than CTL at D60.

3. Lean body mass, bone growth, mineral deposition, and strength at weaning (D21) and puberty (D60) are greater in male rats independent of treatment during early postnatal life.
    1) Percent lean mass measured by DXA at D21 and D60 is greater in male rats impendent of treatment during early postnatal life;
    2) Bone growth indices (bone formation rate by fluorescence labels in tibia shaft and proximal metaphysis) are greater in male rats at D21 and D60 independent of treatment during early postnatal life.
    3) BMC and BMD measured by DXA are greater in male rats at D21 and D60 independent of DMT or CTL during early postnatal life.
    4) Bone strength index (bone microarchitecture by histomorphometry, and Peak Load by three-point bending) are greater in male rats at D21 and D60 independent of treatment during early postnatal life.

Delimitations

The following delimitations were recognized:

1. Animals were randomly grouped at birth to CTL or DMT;
2. Sex and body weight were treated as co-variants in the statistical analyses;

3. Animals were only examined through D0- 60;
4. Animals in the DMT cohort were treated from D 6-10;
5. Animal care tasks were minimized to limit maternal separation for either DMT or CTL cohorts to only 2 minutes each day (D0-21);
6. Duration of separation for MS cohorts was 60 minutes per day from D 6-10;
7. Animals were rotated amongst the dams after each treatment session to control for differences in maternal behavior (licking and grooming, nursing posture) as well as potential variability in the nutrient composition of milk produced by different dams;
8. Animals received identical feeding of standard rat chow from D21-60;
9. Daily rat chow and fluid intake from D21 to 60 were monitored and recorded;
10. Weekly body weight and length were measured and recorded from D0 to 60;
11. Animals were studied at weaning (D21) and early puberty (D60).

Limitations

The following limitations may have influenced the results of this study:

1. Developmental period selected for DMT treatment (D6-10) may not be optimum for desired response;
2. DMT stimulation might be insufficient to elicit desired response;
3. Biochemical markers of bone formation or resorption, glucocorticoid activity, or sex hormones were not included in the analyses;
4. Animals were not studied beyond puberty.

Assumptions

The following assumptions were recognized for this study:

1. All study dams and pups were housed in the same physical environment, and there were no physical activity differences between groups;
2. All study dams and pups in the study were healthy and free of any skeletal deformities or conditions known to interfere with normal bone growth and development;
3. All study dams and pups were not exposed to or influenced by other treatments or interventions.

CHAPTER II

METHODS

This chapter describes the methods that were used to examine the influence of developmental message therapy (DMT) in early postnatal life and its association with weight gain, lean tissue deposition, and skeletal growth in juvenile and young adult rats. This chapter also describes the selection of experimental animals, study protocol, measurements, procedures, and the research design and statistical analyses.

Experimental Animals

Four female, 3-month-old Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing an average of 340g with timed pregnancies were purchased and acclimated to local vivarium conditions. Each rat dam was housed individually (58cm × 36cm × 20cm cage) at room temperature (72°F) with a 12-hour light/12-hour dark cycle. All dams were allowed free access to water and a pelleted commercial natural diet (Teklad Rodent Laboratory Chow #8640, Harlan Teklad, Madison, WI) containing 0.95% calcium, 0.67% phosphorus, and 4.5 IU/g vitamin D_3. Cages were inspected for birth

each morning and the dates of birth was recorded as the previous day unless it was apparent that the litter had recently been born. One day after birth (D1), rat pups were initially weighed and distributed to create weight-matched, sex-balanced litters (n=6; 3M/3F) and assigned to developmental massage therapy (DMT) or control (CTL). Mortality, weights and body size were monitored at intervals throughout the experiment. Rat pups were weaned and separated by sex at age D21. At necropsy (D21 or D60), the animals were anesthetized with Avertin (1ml/100g) and whole blood samples were collected by cardiac puncture, centrifuged and serum frozen for biochemical assay. All animal treatments were conducted according to a University of Utah Institutional Animal Care and Use Committee-approved protocol (#08-4005; Moyer-Mileur, PI), and the animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

Treatment Groups

Two conditions were designed to examine the effects of developmental massage therapy at D21 and D60. The conditions as described throughout the thesis were: (1) CTL for "control control" (dam reared) and (2) DMT for developmental massage therapy (pups were separated from their dam for 10 minutes during which time they were stroked with a soft camelhair brush). The DMT treatment began on D6 and continued daily for 5 days until D10 (n = 6 x 8 = 48 total) in Table 1. Although 5 animals per group was calculated to be sufficient to evaluate a 20% difference in means by an independent samples t-test (2-tailed) with a power of 80%, $\alpha = 0.05$ and $\sigma = 0.10$ (Dupont & Plummer, 1998), in the current study 6 animals were included in each group in order to make up for

Table 1. Experimental design.

Group	Treatment	n
1. D21 CTL Male	No treatment.	6
2. D21 CTL Female		6
3. D60 CTL Male		6
4. D60 CTL Female		6
5. D21 DMT Male	Separated from their dam and stroked with a soft brush for 10 minutes on D6-10.	6
6. D21 DMT Female		6
7. D60 DMT Male		6
8. D60 DMT Female		6

CTL – Control group; DMT - developmental message therapy.

possible losses during the many different steps of the experiment.

Experimental Protocol

Developmental Massage Therapy

This intervention was modified from that originally described by Meaney et al. (1991) and other animal touch/stroking studies (Barnett & Burn, 1967; Bell et al., 1974; Chatterjee et al., 2007; Denenberg et al., 1964; Holst et al., 2005; Kurosawa et al., 1995; Levine, 1957; Lewis, 1959; Lund et al., 2002; Richards, 1966; Smotherman et al., 1977 a, b). DMT was started on D6 and continued daily for 5 days until D10. The pups were removed individually from their home cages, received 10 minutes of gentle brushing of their skin from head to tail in ventral and dorsal positions with a soft camel hair brush. The brush was moistened with warm water at the beginning and through the session to mimic mothers' licking/ grooming. At the end of the DMT session each pup was returned

to its home cage. After all DMT and MS litters had received the intervention the pups were rotated to different dams to decrease the differences in dams' responses (licking/grooming, nursing, or nutrition composition of the milk). Dam and pups of the control group were left entirely undisturbed and never received DMT stimulation during this period except for the common touch for the purpose of daily cage cleaning.

Physical Measurements

The physical indices were recorded in order to detect the possible somatic growth induced by DMT treatment. These measures included body weight after each treatment session, weekly body weight and size (body length of the truck), and daily weight of food and water intake from D21 (weaning) to D60.

Measurements by Peripheral Dual Energy X-ray Absorpitometry (pDXA)

One day prior to necropsy (D20 and D59) bone variables and body composition at the D21 and D60 were measured under isophorene anaesthesia by a peripheral dual energy X-ray absorptiometry (pDXA) (Norland, Medical Systems, Fort Atkinson, WI) (Figure 3). The pDXA has been calibrated for small-animal research to assess body lean mass (g), fat mass (g), bone area (BA, cm^2), bone mineral content (BMC, g), and bone mineral density (areal BMD, g/cm^2) (Grier et al., 1996) in the area of the truck from sternum to coccyx (Figure 4). The daily coefficient of variation for the manufacturer-supplied phantom was 0.6%.

Figure 3. Peripheral dual energy X-ray absorptiometry (pDXA) for BMD, BMC and body composition.

Femur scanned by pDXA provides a measure of areal bone density and has been used as a marker of skeletal adaptation to mechanical loads (Genant et al., 1996). Bone mineral density is a measure of mineral per unit area of bone and does not assess skeletal microarchitecture, which is also a component of bone strength. Because BMD did not accurately correct for changing bone geometry in the growing skeleton of the current study, BMC was the primary outcome variable because it reflects both the material and the geometric properties (Hayes & Bouxsein, 1997). Both BMD and BA are imperfect variables, which only capture the height and width of the bone, without assessing the depth of the bone. However, BMC reflects changes in the true cross sectional area of the skeletal region being examined (Hayes & Bouxsein, 1997)

Figure 4. The area scanned by pDXA for BA, BMC, BMD and body composition

DXA also is a commonly used reference method of showing high reproducibility for body composition assessment which has been used in studies of athletes (Fornetti et al., 1999; Hayes & Bouxsein, 1997; Steward & Hannan, 2000). DXA was originally developed to examine bone mineral density and examines the body by dividing the human body into three parts: bone, fat and bone free (soft) tissue, and fat tissue. The European Society for Clinical Nutrition and Metabolism recommends DXA as a reference method in body composition studies (Kyle et al., 2004).

Bone Strength Detected by Three-point Bending Technique

Three-point bending is useful for measuring the mechanical properties of the bone from rodents and other small animals (Turner & Burr, 1993). Mechanical testing to assess bone strength of the femoral midshafts was carried out on all groups. At the necropsy, the

right femur was wrapped in saline-soaked gauze and immediately frozen and stored at -70°C. The femurs were completely thawed at room temperature 1 hour prior to three-point bending, remaining in the saline gauze. Femur length and diameter of the femoral shaft were recorded using vernier calipers (Mitutoyo, Japan). Femurs were placed under a gradually increasing load until fracture (MTS, Eden Prairie, MN) (Figure 5). The MTS is equipped with a 5-kN load cell and the femur bone was loaded to failure by three-point bending at a loading rate of 10 mm/min (Anonymous, 1992; Hart et al., 2001; Tunner & Burr, 1993). The load was measured with a load cell connected to a computer via an amplifier and the load-deformation curves were recorded online in TestStar IV (MTS Systems Corp., Eden Prairie, MN). Peak load, break load, extrinsic stiffness, and work to fracture were measured from the load-deformation curve (Hart et al., 2001) (Figure 5). Reproducibility of the test was 0.02% of maximum load.

Strain is the percentage change in length or the relative deformation of a material and is reported as a percentage (e.g., strain of .01 = 1% deformation). The stress/strain relationship is represented by a load-deformation curve (Figure 6). The elastic and plastic regions compose the load-deformation curve. Materials in the elastic region will return to their original shape. The elastic region (slope) represents the extrinsic stiffness or rigidity of the bone. The slope of the stress-strain curve within the elastic region is called Young's Modulus (E). Young's Modulus is a measure of intrinsic stiffness of the bone. The slope of this linear region is the nonpermanent deformation. Young's Modulus may be thought of as stiffness or materials resistant to elastic deformation (Rubin & Lanyon, 1985).

Figure 5. Materials testing machine for three-point bending assessment of bone strength.

The plastic region is separated from the elastic region by the yield point. The yield point is a boundary where permanent damage occurs if stresses go above it. The permanent damage is the plastic region of the stress-strain or load-deformation curve (Figure 6).

The force required to break (B) a bone depends on the size of the bone, which is different from intrinsic strength (or Young's Modulus). For example, a drug may decrease the intrinsic strength of a bone, but at the same time increase the bone size; therefore the breaking load remains unchanged (Einhorn, 1994).

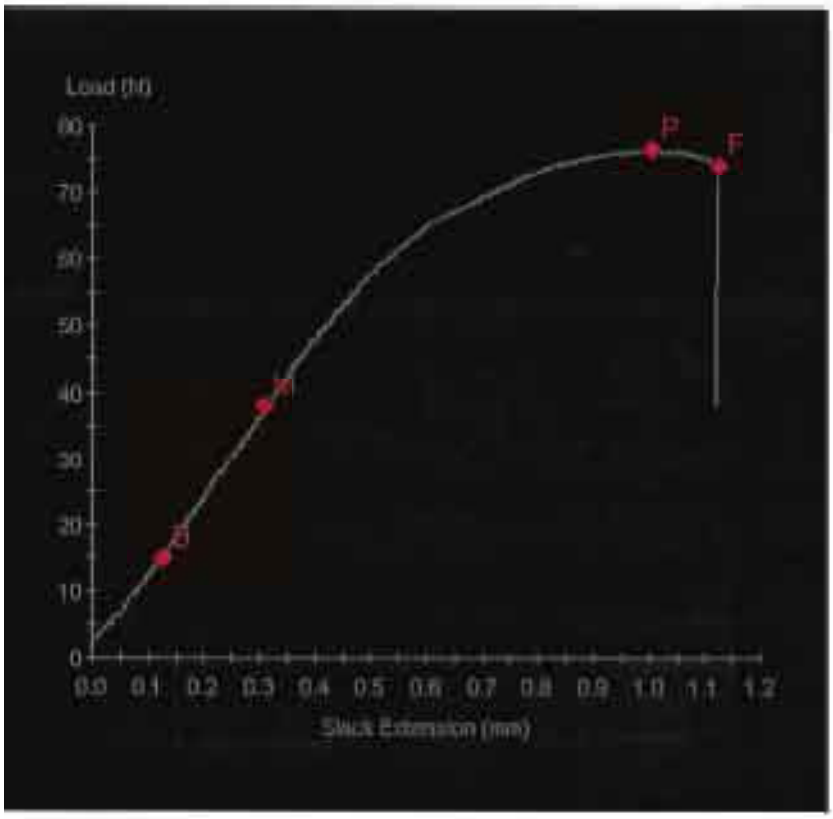

Figure 6. Image from three point bending test showing the Intrinsic Stiffness (slope), Peak Load (P) and Fracture Load (F) by TestStar IV (MTS Systems Corp., Eden Prairie, MN).

Data point for the stress-strain curve can be obtained by interfacing the load cell and strain measurement device with an x-y recorder. From the resulting plot, force, extrinsic stiffness, and Young's modulus can be measured graphically.

Bone Histomorphometry Studies

All experimental pups were injected subcutaneously with 10mg/kg Calcein (Sigma Chemical, St. Louis, MO) on -4 and -1 days prior to necropsy. Calcein is a fluorescent dye with excitation and emission wavelengths of 495/515nm, respectively, and is used for fluorometric determination of calcium. The acetomethoxy derivate of

calcein (Sigma Chemical, St. Louis, MO) is used in bone histomorphometric studies as it is transported through the cellular membrane into live cells, making calcein useful for testing of cell viability and for short-term labeling of bone cells. It has the appearance of orange crystals.

At necropsy the left femur and tibia were harvested and prepared for bone histomorphometric analysis. The tibiae were sliced in half through the mid-diaphyseal shaft and fixed for 24 hours in 0.1 mol/L phosphate buffered formalin. The bone tissues were then dehydrated in ethanol and embedded in methyl methacrylate (Fisher, Los Angeles, CA). Frontal sections of the proximal tibia and cross sections at the tibiofibular junction were cut on a low speed saw (Isomet, Buehler, Lake Bluff, IL), mounted on plastic slides and ground to ~20 μm in thickness using a grinding machine (Exact, Norderstedt, Germany).

Cortical bone analysis. Cortical bone histomorphometric indices were measured at the tibiofibular junction as previously described (Bagi et al., 1993). The primary indices included the total periosteal and endocortical perimeter (mm), bone area (mm^2), marrow area (mm^2), periosteal and endocortical single- and double-labeled surface (sLS & dLS, mm), and interlabel width (μm) (Figure 7). The percentage of cortical area (%Ct.Ar), percentage of mineral surface (%MS), mineral apposition rates (MAR, μm/d) and surface-referent bone formation rates (BFR/BS, $\mu m^3/\mu m^2/d$) were calculated. The histomorphometric nomenclature conforms to recommendations by Parfitt et al. (1983, 1987).

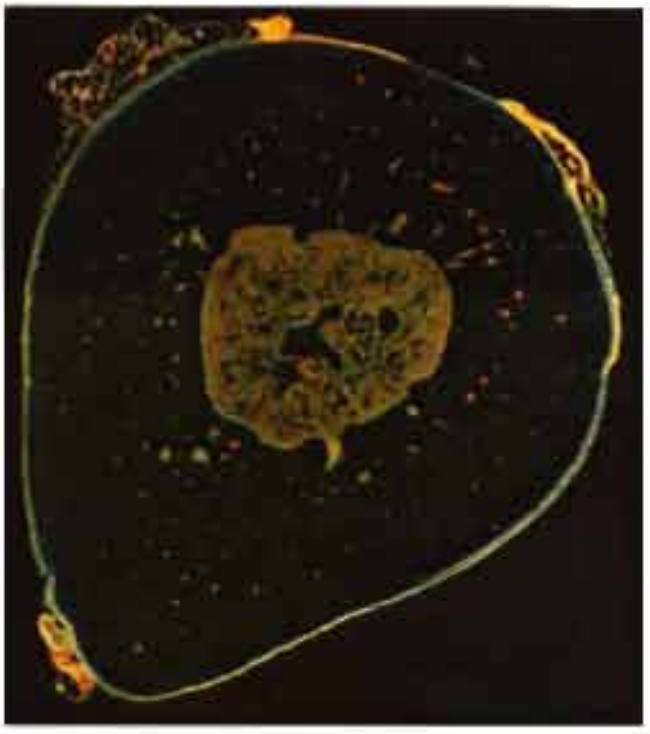

Figure 7. Histomorphometric image of cortical bone on tibia shaft, showing the periosteal / endosteal double labels surface (dLS) and single labels surface (sLS).

Cancellous bone analysis. Microarchitecture and dynamic histomorphometric data were measured in the cancellous bone of the proximal tibial metaphysis, as previously described (Miller & Bowman, 1998). A 2-mm^2 area of cancellous bone in the proximal tibial metaphyseal secondary spongiosa was quantified using a digitizing tablet and a fluorescence microscope (Nikon, Tokyo, Japan) with histomorphometry software (KSS Scientific Consultants, Magna, UT). The proximal boundary of the measured area was defined as the junction of the primary and secondary spongiosa (Figure 8). The primary indices included the total tissue area (mm^2), trabecular bone perimeter (mm), trabecular bone area (mm^2), single- and double-labeled surface (mm), and interlabel width (μm). The percentage of bone area (%B.Ar), trabecular thickness (Tb. Th, μm), number (Tb. N. #/mm) and separation (Tb Sp, μm), and percent resorption (eroded) perimeter (%E.Pm),

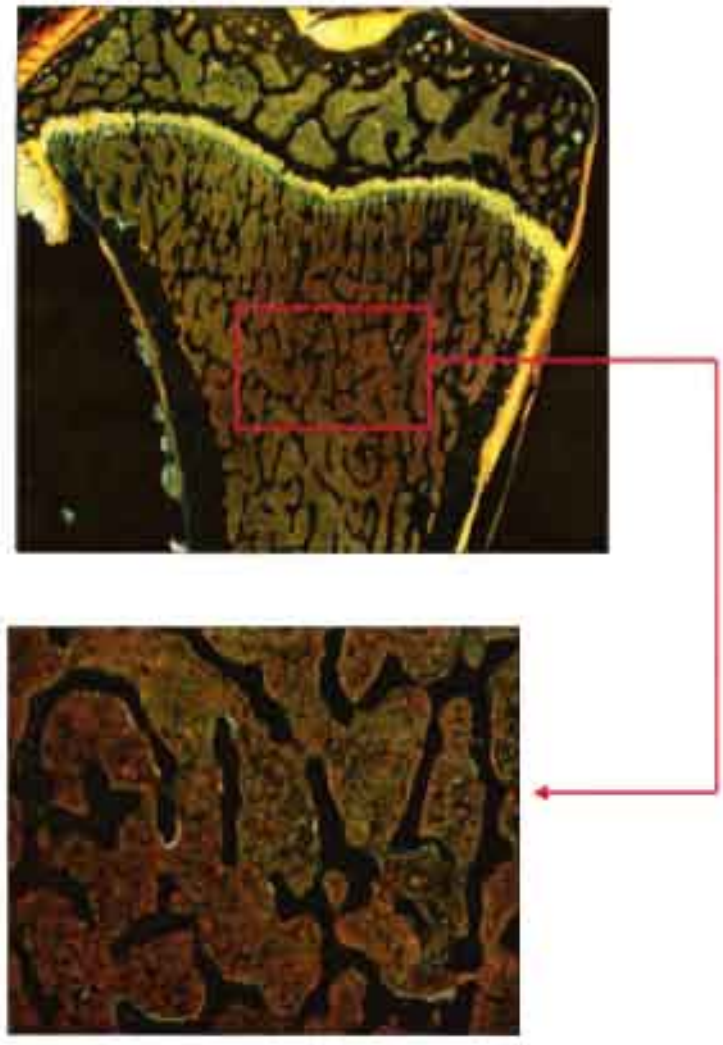

Figure 8. Histomorphometric image of cancellous bone on proximal tibia metaphysis and the measured area.

the percentage of mineralized surface (%MS), mineral apposition rate (MAR, μm/d), and surface-referent bone formation rate (BFR/BS, $\mu m^3/ \mu m^2/d$) were calculated as described by Parfitt et al. (1983, 1987). The mineral apposition rate was corrected for obliquity (Frost, 1983). Trabecular thickness (Tb. Th) is the mean distance across individual trabeculae, in micrometers, and trabecular separation (Tb.Sp) is the mean distance, also in micrometers, between trabeculae. Trabecular number (Tb. N) per millimeter is calculated

as cancellous bone volume/Tb.Th. These variables can be used to evaluate trabecular connectivity and microarchitecture (Parfitt et al., 1983). Eroded surface is the percent of cancellous surface occupied by Howship's lacunae, with and without osteoclasts.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) for each group. Two-tailed bivariate correlations between body weight/soft tissue lean mass and other outcome variables, and ANCOVA adjusted for soft tissue lean mass were performed as appropriate by using SPSS 14.0 package (SPSS 14.0, SPSS Inc. Chicago, Illinois) for the data of body development, pDXA, bone mechanics and histomorphometry at D21 and D60. Overall, main effects for treatment (DMT or CTL) and sex (male or female) and interaction effects for treatment and sex were assessed by two-way ANCOVA. Rationale for using covariates is based on literature identifying soft tissue lean mass/muscle as influential factors on the growing skeleton (Faulkner et al., 1996; Glastre et al., 1990; Kroger et al., 1993; Lu et al., 1994; Slemenda et al., 1991; Zanchetter et al., 1990). Effect size eta^2 and statistical power were limited to the simple effects of treatment variables. Homogeneity of slope was test to meet ANCOVA assumptions. Probabilities (p) less than 0.05 were considered significant.

CHAPTER III

RESULTS

The study was conducted according to the study design and protocol. The main effect of treatment was assessed by ANCOVA and then by two-way ANCOVA to detect treatment and sex interactions. As the DMT intervention did significantly increase body weight and soft tissue lean mass both at D21 and D60, and soft tissue lean mass was treated as a co-variant in the one-way ANCOVA for data analysis. Soft tissue lean mass was also significantly different between sexes and therefore included in the two-way ANCOVA used to evaluate the interaction effects of treatment and sex for both D21 and D60 data. All data are presented as mean ±*SD*.

Developmental Characteristics

The mean body weight at the beginning of the experimental period (D6) was similar for all treatment groups and between males and females (Table 2). Body weight and length, soft tissue lean mass and fat mass results at D21 and D60 by intervention and sex are presented in Table 3.

Table 2. Baseline body weight and length at D6.

	Male		Female	
Basal at D6	Body Weight (g)	Body Length (cm)	Body Weight (g)	Body Length (cm)
D21-CTL	13.26±0.45	5.04±0.19	12.74±0.95	5.06±0.09
D21- DMT	13.19±0.80	5.07±0.31	13.04±0.72	5.03±0.26
D60- CTL	12.54±0.40	5.02±0.11	13.12±0.48	5.04±0.17
D60-DMT	12.52±1.40	4.97+0.19	13.19±0.55	4.96+0.07

*$p<0.05$ vs.CTL

Table 3. Physical development at D21 and D60 by treatment and sex.

	Body Weight (g)	Body Length (cm)	Soft Tissue – Lean Mass (g)	Fat Mass (g)
Male				
D21- CTL	61.31±2.52	8.02±0.08	35.37±1.42	3.04±1.33
D21- DMT	65.05±2.68 *	8.27±0.12 *	49.27±2.89*	3.12±0.90
D60- CTL	342.80±21.95	18.16±0.32	214.78±6.47	5.56±1.97
D60- DMT	360.00±27.93	18.13+0.21	273.19±21.44*	5.72±2.10
Female				
D21- CTL	57.82±1.11	7.96±0.05	35.44±2.45	3.05±1.00
D21- DMT	60.68±3.42	8.15±0.12 *	43.94±2.90*	4.28±2.32
D60- CTL	231.40±7.09	15.96±0.36	161.40±6.41	3.29±0.76
D60- DMT	238.00±8.09	16.00+0.71	175.46±10.97*	2.23±1.62

*$p<0.05$ vs.CTL.

At D21, DMT pups were significantly longer than CTL pups and DMT males heavier than CTL males (+14.5%); there was more soft tissue lean mass found in DMT pups compared to CTL both at D21 (male: +39%, female: +24%) and D60 (male: +27%, female: +9%). No differences were identified for body weight, length or fat mass between groups up to D60. The food and water consumption relative to body weight from D21 to D60 did not differ between the two treatment groups or by sex (data are not shown).

Body Weight or Soft Tissue Lean Mass and Bone

Correlations were performed to identify significant relationships between body weight/soft tissue lean mass and bone size, mineral, density, strength, and histomorphometry studies. The results for body weight and lean mass are presented in Tables 4 and 5.

At D21, male pups' body weight was strongly correlated with periosteal percent mineralized surface and endosteal mineral apposition rates (r=0.70 and 0.84) and moderately related to bone area and mineral content (r=0.60), periosteal mineral apposition rate (r=0.50) and bone surface referent formation rate (r=0.54); whereas the soft tissue lean mass was significantly correlated with bone mineral content (r=0.96), bone area (r=0.92) and femur diameter (r=0.72), and was moderately correlated with periosteal mineral surface (r=0.50). At D21 female pups' body weight was moderately correlated with bone area and bone mineral content (r=0.61 and 0.59) and bone diameter and trabecular separation (r=0.60 and 0.66), whereas the soft tissue lean mass was significantly correlated with bone mineral content (r=0.73), bone area (r=0.75), percent

Table 4. Bivariate correlation analysis between body weight (g) and important bone parameters at D21 and D60.

	Male		Female	
	D21 (n=12)	D60 (n=12)	D21 (n=12)	D60 (n=12)
BMD(g/cm^2)	0.26	0.41	0.11	-0.27
BMC(g)	0.60	0.60*	0.59	0.62
BA (cm^2)	0.60	0.43	0.61*	0.59
%Ct. Ar	0.14	-0.18	-0.49	0.26
P.%MS	0.70*	0.09	0.73*	-0.17
P.MAR (μm/d)	0.54	-0.05	0.20	0.62
P.BFR/BS (μm3/μm2/d)	0.50*	-0.08	0.36	0.53
E.%MS (%)	-0.33	0.52	0.20	0.29
E.MAR (μm/d)	0.84*	-0.48	0.51	-0.16
E.BFR/BS (μm3/μm2/d)	0.31	-0.14	0.50	-0.09
Erosion Surface (%)	-0.46	-0.33	0.58	0.19
Tb.Wi (μm)	-0.23	-0.08	0.14	0.41
Tb. N (#/mm)	0.07	-0.23	-0.57	0.08
Tb.Sp (μm)	-0.20	-0.03	0.60	-0.15
T.% MS (%)	-0.28	0.37	0.44	0.37
T.MAR (μm/d)	-0.09	0.33	-0.29	0.36
T.BFR/BS (μm3/μm2/d)	-0.15	0.31	0.17	0.34
Diameter (mm)	0.15	-0.30	0.66*	0.39
Length (mm)	0.34	-0.15	0.54	0.16
Peak Load (N)	0.17	-0.01	-0.06	0.38
Intrinsic Stiffness (N/mm)	0.01	0.27	0.15	0.13

%Ct.Ar: percent cortical area;P.MAR: periosteal mineral apposition rate; P.BFR/BS: periosteal bone surface referent bone formation rate; E.%MS: endosteal percent mineralized surface; E.MAR: endosteal mineral apposition rate; E.BFR/BS: endosteal bone surface referent bone formation rate; Tb.Wi: trabecular width; Tb.N: trabeculea number; Tb.Sp: trabecular separation; T.%MS: trabecular percent mineralized surface; T.MAR: trabecular mineral apposition rate; T.BFR/BS: trabecular bone surface referent bone formation rate.

*$p<0.05$, two tailed.

Table 5. Bivariate correlation analysis between soft tissue lean mass (g) and important bone parameters at D21 and D60.

	Male		Female	
	D21 (n=12)	D60 (n=12)	D21 (n=12)	D60 (n=12)
BMD(g/cm^2)	0.45	0.16	0.41	0.22
BMC(g)	0.96*	0.90*	0.73*	0.44
BA (cm^2)	0.92*	0.79*	0.75*	0.67*
%Ct. Ar	0.16	-0.09	0.67*	0.22
P.%MS	0.50	0.16	0.29	0.07
P.MAR (μm/d)	0.11	0.41	0.02	0.61
P.BFR/BS (μm3/μm2/d)	0.19	0.17	0.05	0.61
E.%MS (%)	-0.21	0.70*	-0.01	0.57
E.MAR (μm/d)	0.48	0.63*	0.73*	-0.07
E.BFR/BS (μm3/μm2/d)	0.12	0.10	0.29	0.21
Erosion Surface (%)	-0.23	-0.10	-0.40	-0.12
Tb.Wi (μm)	0.49	0.43	0.42	0.78*
Tb. N (#/mm)	0.10	-0.10	-0.10	0.18
Tb.Sp (μm)	-0.20	-0.14	-0.09	-0.49
T.% MS (%)	0.46	0.44	-0.05	0.24
T.MAR (μm/d)	0.21	0.73*	0.38	0.49
T.BFR/BS (μm3/μm2/d)	0.34	0.60	0.15	0.36
Diameter (mm)	0.72*	0.11	0.68*	0.28
Length (mm)	0.44	0.18	0.44	0.42
Peak Load (N)	0.15	0.15	0.20	0.39
Intrinsic Stiffness (N/mm)	-0.05	0.64*	0.38	0.36

%Ct.Ar: percent cortical area;P.MAR: periosteal mineral apposition rate; P.BFR/BS: periosteal bone surface referent bone formation rate; E.%MS: endosteal percent mineralized surface; E.MAR: endosteal mineral apposition rate; E.BFR/BS: endosteal bone surface referent bone formation rate; Tb.Wi: trabecular width; Tb.N: trabeculea number; Tb.Sp: trabecular separation; T.%MS: trabecular percent mineralized surface; T.MAR: trabecular mineral apposition rate; T.BFR/BS: trabecular bone surface referent bone formation rate; .

*$p<0.05$, two tailed.

cortical area (r=0.67), endosteal mineral apposition rate (r=0.73) and femur diameter (r=0.68).

At D60, male body weight was moderately correlated to bone mineral content (r=0.60) and endosteal percent mineralized surface; the soft tissue lean mass was significantly correlated with bone mineral content (r=0.90), bone area (r=0.79), percent endosteal mineral surface (r=0.70), endosteal (r=0.63) and trabecular mineral apposition rate (r=0.73), and femur intrinsic stiffness (r=0.64), and was moderately correlated with trabecular bone formation rate (r=0.60). Female body weight was moderately related to bone area and mineral content (r=0.59 and 0.62) and perisoteal mineral apposition rate (r=0.62) and periosteal bone surface referent bone formation rate (r=0.52); the soft tissue lean mass was significantly correlated with bone area (r=0.67) and trabecular thickness (r=0.78), and was moderately correlated with periosteal mineral apposition rate (r=0.61), periosteal bone formation rate (r=0.61) and endosteal percent mineral surface (r=0.57).

Bone Area, Bone Mineral Density and Bone Mineral Content

The DXA-derived BA, BMC, BMD, percent lean mass and percent fat mass findings are shown in Table 6. At D21, BMD was significantly higher in DMT pups than CTL pups after adjusting for soft tissue lean mass. No differences were detected for bone area after adjusting for soft tissue lean mass except that D60 DMT males had larger bone area versus CTL males (+39%). No differences were detected for BMC after adjusting for soft tissue lean mass between groups at either D21 or D60.

Table 6. BA, BMC and BMD at D21 and D60 by treatment and sex.

	BA (cm^2)	BMC (g)	BMD (g/cm^2)
Male			
D21- CTL	7.42±0.73	0.34±0.04	0.046±0.003
D21- DMT	11.72±0.81	0.56±0.05	0.048±0.004*
D60- CTL	18.09±0.41	2.43±0.10	0.134±0.004
D60- DMT	25.11±2.43*	3.29±0.30	0.134±0.008
Female			
D21- CTL	7.86±1.74	0.41+0.10	0.047±0.005
D21- DMT	11.19±1.57	0.53±0.06	0.048±0.002*
D60- CTL	19.12±0.99	2.61±0.14	0.137±0.006
D60- DMT	19.12±1.63	2.60±0.25	0.132±0.007

CTL - Control group; DMT - developmental message therapy. BA: bone area; BMC: bone mineral content; BMD: bone mineral density.

*$p<0.05$ vs.CTL. Adjusted for soft tissue lean mass.

Mechanical Testing

Results from the three-point bending test for femur shafts are shown in Table 7. At D21, the diameter of the femur shaft was 14% greater in DMT females than CTL. At D60, the diameter and length of the femur shaft were significantly greater in DMT males compared to CTL males; DMT females were found to have increased peak load (16%) when compared to CTL females.

Tibia Shaft and Metaphysis Histomorphometric Profile

Tibia Shaft Cortical Bone

A trend toward greater periosteal mineral apposition rate and bone formation rate was identified in D21 DMT pups although these differences were not statistically significant by treatment or sex (Table 8). However, D60 DMT females demonstrated an increased percent mineral surface on the endosteal surface when compared to CTL females (+13%).

Microarchitecture in the Primary Tibia Metaphysis

No differences were found between treatments or by sex at D21 or D60 after adjusting for soft tissue lean mass. However, there were trends for trabecular width to be greater among D60 DMT males than in CTL males, and DMT females tended to have decreased trabecular separation when compared to CTL females (Table 9).

Table 7. Bone mechanics variables for the femur midshaft at D21 and D60.

	Diameter (mm)	Length (mm)	Peak Load (N)	Intrinsic Stiffness (N/mm)
Male				
D21- CTL	2.46±0.10	16.85±0.99	21.43±2.51	63.26±15.75
D21- DMT	2.70±0.14	17.60±0.57	20.55±3.15	60.42±9.90
D60- CTL	4.45±0.15	32.37±0.41	81.88±4.72	120.74±13.77
D60- DMT	4.66±0.30*	32.88±0.53*	85.98±7.20	148.77±18.20
Female				
D21- CTL	2.42±0.09	17.26±0.18	23.04±2.18	76.66±6.77
D21- DMT	2.77±0.13*	17.50±0.34	21.38±2.68	70.05±10.62
D60- CTL	3.89±0.12	30.13±0.59	75.11±3.48	152.86±18.77
D60- DMT	4.21±0.27	30.87±0.78	87.15±5.26*	171.13±27.51

CTL - Control group; DMT - developmental message therapy; Diameter: femur shaft diameter; Length: femur shaft length.

*$p<0.05$ vs.CTL. Adjusted for soft tissue lean mass.

Table 8. Cortical bone histomorphometric variables of the tibia shaft at D21 and D60.

	Periosteal Surface				Endosteal Surface		
	%Ct.Ar (%)	%MS (%)	MAR (μm/d)	BFR/BS ($\mu m^3/\mu m^2/d$)	%MS (%)	MAR (μm/d)	BFR/BS ($\mu m^3/\mu m^2/d$)
Male							
D21-CTL	54.91±2.06	98.55±0.90	19.25±2.76	16.48±5.29	35.26±8.47	5.06±0.7	1.78±0.44
D21- DMT	55.39±1.91	96.31±2.60	21.02±4.24	19.69±3.46	32.96±7.65	5.76±0.73	1.94±0.54
D60-CTL	78.85±1.94	93.06±1.43	9.24±1.61	9.11±0.93	74.70±3.36	4.68±0.59	3.47±0.40
D60- DMT	78.83±3.16	93.27±3.02	11.26±1.99	10.31±2.04	81.82±7.11	4.39±0.57	3.67±0.71
Female							
D21-CTL	51.77±1.82	95.01±2.15	17.85±2.56	16.98±2.63	36.61±6.8	5.05±0.81	1.82±0.41
D21- DMT	51.07±0.88	96.19±5.05	18.85±5.40	17.92±5.24	38.57±10.46	6.17±0.27	2.36±0.89
D60- CTL	76.76±1.80	95.98±3.20	8.55±1.34	8.44±1.28	81.30±3.84	4.61±0.86	3.82±0.51
D60- DMT	75.21±2.18	96.62±2.95	9.11±2.33	8.75±2.15	92.84±3.75*	4.28±0.91	4.03±1.03

CTL - Control group; DMT - developmental message therapy; %Ct.Ar: percent cortical area = (total area – marrow area)/total area *100; %MS: percent mineralized surface = (dLS + sLS/2)/ periosteal or endosteal perimeter * 100; MAR: mineral apposition rate= interlabel width / days; BFR/BS: bone surface referent bone formation rate MS * MAR / periosteal or endosteal perimeter.

*$p<0.05$ vs.CTL. Adjusted for soft tissue lean mass.

Table 9. Cancellous bone microarchitectural variables in tibia metaphysis at D21 and D60.

	%B.Ar (%)	Tb.Wi (μm)	Tb.N (#/mm)	Tb.Sp (μm)
Male				
D21- CTL	12.83±4.49	47.39±6.93	2.55±0.76	362.23±109.95
D21- DMT	11.45±2.74	42.33±3.15	2.71±0.65	329.81±71.03
D60- CTL	17.16±3.72	49.21±4.03	3.58±0.63	270.19±81.21
D60-DMT	19.98±6.97	59.24±8.80	3.45±0.96	242.65±70.94
Female				
D21- CTL	10.23±2.61	36.13±4.75	2.83±0.57	328.73±74.33
D21-DMT	10.00±1.71	35.11±2.44	2.78±0.51	336.02±82.19
D60- CTL	21.53±3.39	55.30±8.85	4.17±0.45	210.86±39.09
D60-DMT	26.96±5.70	59.23±12.87	4.58±0.82	165.29±47.33

CTL - Control group; DMT - developmental message therapy. %B.Ar: percent trabecular area = bone area / total tissue area * 100; Tb.Wi: trabecular width = (2000/1.199)* bone area / bone perimeter; Tb.N: trabeculea number = (1.199/2)* bone perimeter / tissue area; Tb.Sp: trabecular separation = (2000/1.199)* (tissue area – bone area) / bone perimeter.

*$p<0.05$ vs.CTL. Adjusted for soft tissue lean mass.

Histomorphometric Profile of Trabecular Bone in Primary Tibia Metaphysis

At D21, percent mineral surface, mineral apposition rate and bone formation rate increased on the cancellous bone surface after adjusting for soft tissue lean mass. No differences were detected statistically for trabecular bone variables in the tibia metaphysis up to D60 by treatment; although there was a trend toward greater increases in mineralization and bone formation in the DMT pups (Table 10).

Sex and Treatment Interactions

Results for the two-way ANCOVA test to determine the main effect for sex and the interaction effects between treatment and sex are displayed in Table 11. At D21, male pups had greater cortical bone area in the tibia shaft and thicker trabeculae in the primary tibia metaphysis; there was higher intrinsic stiffness found in female pups at D21. Up to D60, female pups had greater endosteal mineral surface in the tibia shaft. Bone mineral content, bone area and the femur shaft diameter and length were greater in D60 males compared to females. A significant treatment and sex interaction effect was detected for bone mineral content and bone area at D60 (Figure 9).

Effect Size and Statistical Power

There are six eta^2 values above the threshold of 0.06 for a "medium" effect (Cohen, 1988) among the nonsignificant effects of D21 male rats (bone area, periosteal mineral apposition rate, periosteal bone formation rate, trabecular mineral surface, trabecular bone formation rate and femur intrinsic stiffness), yet the statistical power

Table 10. Cancellous bone histomorphometric variables in tibia metaphysis at D21 and D60.

	%E.Pm (%)	%MS (%)	MAR (μm/d)	BFR/BS ($\mu m^3/\mu m^2/d$)
Male				
D21- CTL	6.52±1.37	39.03±6.35	3.93±0.34	1.18±0.26
D21- DMT	6.04±1.19	35.27±4.72	3.80±0.45	1.07±0.20
D60- CTL	6.94±1.77	38.63±2.92	4.23±0.17	1.35±0.12
D60-DMT	6.56±2.47	42.33±5.08	5.01±0.40	1.69±0.33
Female				
D21- CTL	6.26±1.51	38.12±4.70	3.67±0.56	1.07±0.26
D21-DMT	7.91±0.65	40.46±3.91*	3.63±0.27*	1.16±0.13*
D60- CTL	5.05±2.81	33.31±3.18	4.73±0.48	1.31±0.24
D60-DMT	5.26±1.23	38.90±5.38	5.33±0.81	1.68±0.41

CTL - Control group; DMT - developmental message therapy; %E.Pm: percent eroded perimeter = eroded perimeter / bone perimeter * 100; %MS: percent mineralized surface = (dLS + sLS/2)/ bone perimeter * 100; MAR: mineral apposition rate= interlabel width / days; BFR/BS: bone surface referent bone formation rate MS * MAR / bone perimeter.

*$p<0.05$ vs.CTL. Adjusted for soft tissue lean mass.

Table 11. Significant main effects of sex at D21 and D60 assessed by two-way ANCOVA.

D21	Sex main effect *p* value
%Ct. Ar	< 0.001
Tb.Wi (µm)	<0.001
Intrinsic Stiffness (N/mm)	0.035
D60	
BMC(g)	0.015
BA(cm^2)	0.002
E.%MS	0.018
Femur Diameter (mm)	0.002
Femur Length (mm)	0.002

%Ct.Ar: percent cortical area; E.MAR: endosteal mineral apposition rate; Tb.Wi: trabecular width; E.%MS: endosteal percent mineralized surface; BMC: bone mineral content; BA: bone area by pDXA.

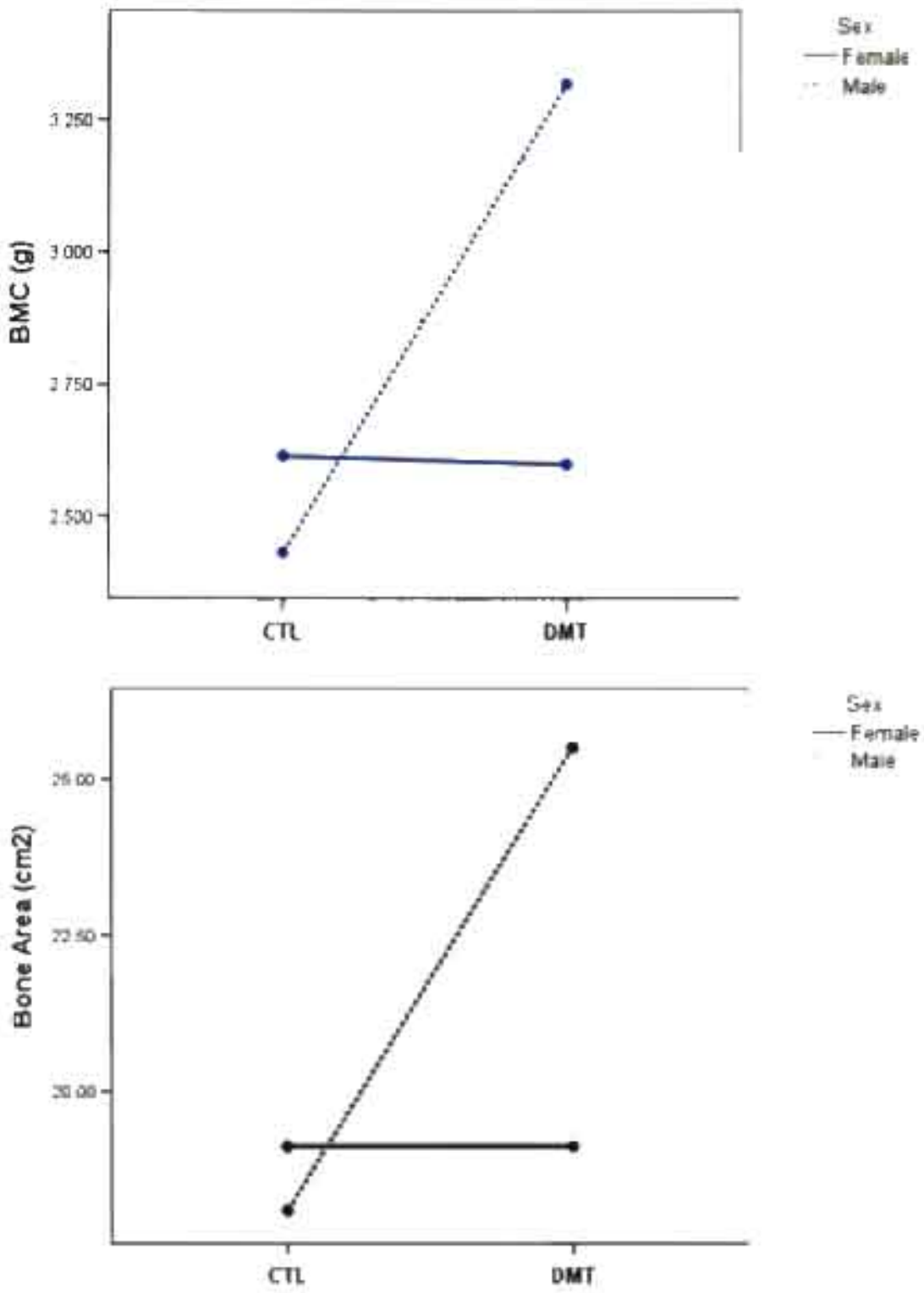

Figure 9. The plots for the significant interaction effects of bone mineral content and bone area at D60 ($p<0.05$).

ranged from only 19% to 50%, and 11 such eta^2 values in D60 male rats (bone mineral density, bone mineral content, periosteal mineral surface, periosteal mineral apposition rate, periosteal bone formation rate, endosteal mineral apposition rate, endosteal bone formation rate, trabecular thickness, trabecular mineral apposition rate, femur peal load and intrinsic stiffness) and the statistical power ranged from only 14% to 38% (Table 12).

In the female rats, at D21 there are also six eta^2 above 0.06 among these nonsignificant effects (tibia shaft cortical area, endosteal mineral surface, endosteal bone formation rate, erode surface, trabecular thickness and femur peak load) and the statistical power ranged from only 11% to 48%; and at D60 there are 15 such eta^2 values (bone mineral density, bone mineral content, bone area, tibia shaft cortical area, periosteal mineral apposition rate, periosteal bone formation rate, endosteal mineral apposition rate, trabecular thickness, trabecular number, trabecular separation, trabecular mineral apposition rate, trabecular bone formation rate, femur diameter, length and intrinsic stiffness) and the statistical power ranged from only 13% to 45% (Table 13).

Table 12. Effect size and power of important bone variables in ANCOVA analysis for male rats at D21 and D60.

	D21		D60	
	Effect size (eta^2)	Power (%)	Effect size (eta^2)	Power (%)
BMD(g/cm^2)	0.78*	99	0.12	17
BMC(g)	0.01	6	0.23	31
BA (cm^2)	0.37	48	0.48*	72
%Ct. Ar	0.04	8	0.03	7
P.%MS	0.01	5	0.13	16
P.MAR (μm/d)	0.23	27	0.16	19
P.BFR/BS ($μm^3/μm^2/d$)	0.15	19	0.27	33
E.%MS (%)	<0.01	5	0.01	5
E.MAR (μm/d)	<0.01	5	0.27	33
E.BFR/BS ($μm^3/μm^2/d$)	<0.01	5	0.19	23
Erosion Surface (%)	0.02	6	<0.01	5
Tb.Wi (μm)	<0.01	5	0.30	38
Tb. N (#/mm)	0.02	7	<0.01	5
Tb.Sp (μm)	<0.01	5	0.03	7
T.% MS (%)	0.39	50	0.01	6
T.MAR (μm/d)	0.04	8	0.24	29
T.BFR/BS ($μm^3/μm^2/d$)	0.27	33	0.03	8
Diameter (mm)	<0.01	5	0.49*	68
Length (mm)	0.04	8	0.53*	75
Peak Load (N)	0.02	6	0.20	24
Intrinsic Stiffness (N/mm)	0.17	21	0.11	14

%Ct.Ar: percent cortical area;P.MAR: periosteal mineral apposition rate; P.BFR/BS: periosteal bone surface referent bone formation rate; E.%MS: endosteal percent mineralized surface; E.MAR: endosteal mineral apposition rate; E.BFR/BS: endosteal bone surface referent bone formation rate; Tb.Wi: trabecular width; Tb.N: trabeculea number; Tb.Sp: trabecular separation; T.%MS: trabecular percent mineralized surface; T.MAR: trabecular mineral apposition rate; T.BFR/BS: trabecular bone surface referent bone formation rate; .

*Statistically significant in ANCOVA analysis.

Table 13. Effect size and power of important bone variables in ANCOVA analysis for female rats at D21 and D60.

	D21		D60	
	Effect size (eta^2)	Power (%)	Effect size (eta^2)	Power (%)
BMD(g/cm^2)	0.54*	76	0.15	16
BMC(g)	<0.01	5	0.24	25
BA (cm^2)	0.06	10	0.13	13
%Ct. Ar	**0.12**	13	0.42	49
P.%MS	**0.05**	8	<0.01	5
P.MAR ($\mu m/d$)	**0.06**	9	0.20	21
P.BFR/BS ($\mu m^3/\mu m^2/d$)	**0.03**	7	0.35	39
E.%MS (%)	**0.08**	11	0.57*	75
E.MAR ($\mu m/d$)	**0.06**	9	0.20	21
E.BFR/BS ($\mu m^3/\mu m^2/d$)	**0.10**	12	0.02	6
Erosion Surface (%)	**0.41**	48	<0.01	5
Tb.Wi (μm)	**0.30**	32	0.18	19
Tb. N (#/mm)	0.01	6	0.34	37
Tb.Sp (μm)	<0.01	5	0.39	45
T.% MS (%)	0.55*	70	0.33	36
T.MAR ($\mu m/d$)	0.46*	56	0.04	7
T.BFR/BS ($\mu m^3/\mu m^2/d$)	0.65*	87	0.16	17
Diameter (mm)	0.57*	82	0.33	37
Length (mm)	**0.01**	5	0.13	14
Peak Load (N)	0.15	19	0.79*	99
Intrinsic Stiffness (N/mm)	0.01	6	0.15	17

%Ct.Ar: percent cortical area;P.MAR: periosteal mineral apposition rate; P.BFR/BS: periosteal bone surface referent bone formation rate; E.%MS: endosteal percent mineralized surface; E.MAR: endosteal mineral apposition rate; E.BFR/BS: endosteal bone surface referent bone formation rate; Tb.Wi: trabecular width; Tb.N: trabeculea number; Tb.Sp: trabecular separation; T.%MS: trabecular percent mineralized surface; T.MAR: trabecular mineral apposition rate; T.BFR/BS: trabecular bone surface referent bone formation rate; .

*Statistically significant in ANCOVA analysis.

CHAPTER IV

DISCUSSION

This purpose of this study was to test the impact of DMT during early postnatal life on growth and skeletal development in an animal model. This is the first investigation to describe the effects of DMT on body composition and bone mineral content, strength and histomorphometry in juvenile and young adult rats. Consistent with the previous clinical studies (Field, 2005; Litmanovitz et al., 2003, 2004, 2007; Moyer-Mileur et al., 1995, 2000, 2008; Nemet et al., 2002), the current study demonstrated that a DMT intervention during early life was associated with greater weight gain, soft tissue lean mass, and bone growth, mineral acquisition, and strength in juvenile and young adult rats.

Body weight and length at weaning (D21) were greater in both male and female rat pups after receiving daily DMT during early postnatal life (D6-D10) (Table 3). These growth differences are attributed to the DMT intervention as litter variation was minimized by cross-fostering to control for maternal behavior and/or nutrient composition of dam milk, and all animals were housed in the same environment throughout the study period. Animal and human studies of stress during the neonatal

period have reported an inverse association between weight gain and levels of cortisol or epinephrine (Fenoglio et al., 2006; Field et al. 1986, 2002, 2005, 2006; Jutapakdeegul et al., 2003; Moyer-Mileur et al., 2008; Panagiotaropoulos et al., 2004; Phillips & Jones, 2006). It is postulated that massage modulates the autonomic nervous system's response and recovery to environmental stressors thus minimizing the negative impact of stress on postnatal growth (Fenoglio et al., 2006; Field et al., 1986, 2002, 2005, 2006; Jutapakdeegul et al., 2003; Moyer-Mileur et al., 2008; Panagiotaropoulos et al., 2004; Phillips & Jones, 2006). In premature infants, massage including touch with kinesthetic movement, has been linked to decreased cortisol levels, lower blood pressure, and increased gastric motility and the release of GI hormones (Field et al., 1986, 2002, 2005, 2006). Therefore, the positive effects of DMT on growth may be the result of an improved neuroendocrine system response to stress, which allows more efficient absorption of energy and nutrients required for growth.

To our knowledge, this is the first investigation of DMT intervention applied to the normal postnatal animal model without neonatal stress involved. The previous studies mentioned above are all based on a neonatal stress model either in clinical or animal research, such as premature or mother-separation conditions. Our study added the understanding of the efficacy of DMT that improved the postnatal development even without a preexisting disordered or impaired neuroendocrine system.

This study also demonstrated greater soft tissue lean mass in DMT pups independent of sex at weaning (D21 male 39% greater and female 24% greater than D21 CTL) and young adulthood (D60 male 27% greater and female 9% greater than D60 CTL) (Table 3). As previously noted, stress increases circulating cortisol levels. Chronically

elevated cortisol levels are known to alter metabolism and body composition by increasing soft tissue lean mass catabolism and intra-abdominal fat stores. The strong relationship between body weight and soft tissue lean mass (r=0.72) and greater growth provides support for modulation of the neuroendocrine system's stress response in the DMT cohort.

Body weight and soft tissue lean mass were associated with higher bone formation and mineral content in this study (Tables 4 & 5). Body weight was significantly correlated with periosteal mineral surface, periosteal bone formation rate, endosteal mineral apposition rate in male rats, and with DXA derived bone area, periosteal mineral surface and femur diameter in females. Whereas soft tissue lean mass was significantly correlated with bone mineral content, DXA derived bone area, endosteal mineral surface, endosteal mineral appostion rate, cancellous bone mineral apposiotion rate, femur diameter and intrinsic stiffness in male rats, and with bone mineral content, DXA derived bone area, tibia shaft cortical bone area, endosteal mineral apposition rate, trabecular thickness and femur diameter in females. Epidemiological investigations of the relationship between body weight or body mass index (wt/ht^2) and bone mass have produced the generally accepted view that increased body weight increases the mechanical load on bone which, in turn, contributes to increases in bone mass (Fassler & Bonjour, 1995; Skerry et al., 2003; Slemenda et al., 1994;). Bone mass accrual is tightly controlled by mechanical loads or "strain" on bone generated body from muscle forces (Frost, 1996, 1997). Mechanical strain is defined as a change in dimensions and/or shape of the bone in response to forces exerted by soft tissue lean mass or body weight on bone. During periods of rapid growth, both body weight and muscle forces

exert a load on the skeleton that causes mechanical strain. Throughout life, mechanical strain appears to be an important signaling mechanism in bone by helping to control structural adaptations to the mechanical use (Frost, 1996). For example, mechanical strain between 800 and 1600 microstrains (μE) preserves bone, whereas mechanical strain that regularly exceeds 1600 μE promotes greater bone strength (Frost, 1996).

The recognition of the skeletal system's ability to adapt throughout the lifecycle was originally described by Wolff (1870, 1899). Wolff's Law was redefined by Frost (1990 a, b) with the inclusion of mechanical strain on bone as the driving force required for structural adaptation of the skeleton. Frost proposed a negative feedback system or "mechanostat" to explain how forces affect the shape of bones. Additional work by Schiessl et al. (1998) identified muscle contraction as the source of musculoskeletal forces. Whereas others had observed correlations between measures of muscle and bone tissues before (Jones et al., 1983), these authors were the first to discuss the muscle-bone unit as a functional symbiosis.

The study of pediatric bone growth was the first to provide substantial evidence to the muscle-bone hypothesis. In children, both bone and muscle mass increase linearly until puberty (Neu et al., 2001; Schoenau et al., 2004; Zanchetta et al., 1995) and there are no sex differences in BMC or BMD by DXA in prepubertal children (Nelson et al., 1997; Nguyen et al., 2001). At age 12, girls begin to experience bone mass accretion that exceeds muscle mass. However, a similar finding was not observed in adolescent boys (Neu et al., 2001; Schoenau et al., 2004; Zanchetta et al., 1995). Nguyen et al. (2001) reported that BMD values during puberty in girls were higher in the hip and spine regions, whereas BMD values in the whole skeleton were higher in postpubertal boys.

Schonau et al. (1996) used peripheral quantitative computed tomography (pQCT) of the radius to document the close relationship between muscle and bone mass in children. These investigators showed that muscle development precedes bone development by 2 to 3 months suggesting a causal role for the accrual of bone strength throughout childhood and adolescence. Interestingly, this muscle-bone relationship seems to be shifted in girls after puberty (Schonau et al., 2000), with women in their reproductive years having more bone mass being explained by their muscle strength.

Greater bone dimensions in DMT rat pups were further verified by physical measures of the femur size and bending strength (Tables 6 & 7). A significantly larger femur midshaft diameter and length was found at D60 in DMT male rat pups and was positively correlated with DXA-derived bone area (r =0.91). Although no differences in femur bone strength were detected at weaning we did find significantly greater femur bone strength in D60 DMT females. The absence of a similar finding in D60 DMT males may reflect differences in the timing and tempo of pubertal-driven skeletal growth and expansion (Hefferen et al., 2003), with puberty achieved at an earlier age in female rats than in males. More specifically, sexual maturation is attained at the beginning of D34 of life in female rats whereas the males do not complete pubertal-driven skeletal growth and maturation until D65.

Although body size and muscle force have been identified as major contributing factors for skeletal growth and development, other factors must also be considered. Somatic growth is controlled by pituitary gland-derived growth hormone (GH), which stimulates insulin-like growth factor 1 (IGF1) activity in the liver, bone, muscle, and other tissues. The GH/IGF1 axis activity is greatest during the early postnatal period and

again during adolescence. Physical and environmental stressors have been shown to diminish GH/IGF1 activity during critical periods of growth and development in infants and young animals. Higher IGF1 levels and bone mineral deposition have been reported in premature infants who received a daily DMT during early life (Moyer-Mileur et al. 2008). Although positive effects of DMT on soft tissue lean mass acquisition and subsequent greater bone mineral acquisition were documented, other factors or the mechanism(s) responsible for this finding were not explored in the current study. Thus, exploration of the association of DMT and GH/IGF1 may help define mechanisms for DMT and weight gain, body composition, and skeletal growth and development.

Interestingly, D60 DMT male rats were larger and had greater femur bone size whereas D60 DMT females displayed higher peak load in the three-point bending test (Tables 7 & 8). Periosteal bone formation can be a compensatory mechanism to maintain bone strength in situations where bone loss occurs from trabecular and endocortical surfaces (Alhlborg et al., 2003). Periosteal expansion significantly increases bone strength (Allen et al., 2004). The relationship between periosteal dimension and bone strength is exponential; increases of periosteal radii enhance modulus (an estimator of bone strength) by the fourth power (Orwoll, 2003). Small increases in periosteal apposition are mechanically advantageous as limited amounts of new bone can substantially increase fracture resistance and can mechanically offset loss of endocortical/trabecular bone. Estrogen has surface-specific effects on cortical bone; on the periosteal surface it inhibits the proliferation and differentiation of osteoblasts, whereas on the endocortical surface it might enhance osteoblastic activity (Garn, 1970,

1972). Periosteal bone formation is largely due to the muscle-bone relationship, in which muscle contributes to the largest load-bearing effect (Jee, 2000; Kalu et al., 2000).

The bone histomorphometry studies support and provide insight into the previously addressed bone growth and mineral gains observed in the DMT animals. Specifically, DMT during early postnatal life increased the endosteal mineral surface on cortical bone area, and the mineralized surfaces, mineral apposition and bone formation rates for cancellous bone surface in juvenile and young adult rats (Tables 8, 9 & 10).

Histomorphometric evaluation of the tibia and femur bones confirmed the presence of the modeling drift that occurs during rapid skeletal expansion and mineral deposition (Tunner et al., 1994). During modeling the trabecular bone microarchitecture is improved by cancellous bone formation and consolidation. Indeed, the increased percent mineral surface, mineral apposition and bone formation rates on the cancellous bone surface at D21 DMT female rats (Table 10), as well as the greater percent endosteal mineralized surface and the trend of decreased trabecular spacing, confirms the development of a thicker trabeculae in D60 DMT females (Table 9). A trend of increased trabecular bone area or density enhances bone strength, and greater bone strength was also found in D60 DMT females (Tables 7 &9). Conversely, D60 DMT males exhibited a tendency of greater trabecular width and mineral apposition rate compared to D60 CTL males (Table 9). It appears that DMT impacted histomorphometric variables in females during juvenile period (D21) in a more sensitive manner than in males, and then developed a prolonged, positive influence of DMT on bone strength at young age (D60). The absence of similar findings in DMT males may be explained by puberty. During puberty the sex steroids estrogen and testosterone drive accelerated soft tissue lean mass

and skeletal growth by increasing GH/IGF1 axis activity. In rats, the onset of puberty occurs at a later age in males (D45) compared to females (D34) (Tinwell et al., 2002). Therefore, sex differences in bone tissue development are most likely due to the timing of the onset of puberty.

During puberty, sex hormones induce an increase in the GH/IGF system to promote linear growth and bone expansion. As maturation progress, bone turnover is reduced, which increases cortical bone thickness and strength. An early study of cortical dimensions, based on two-dimensional radiogrammetry, concluded that a greater cortical bone mass in healthy boys was caused by sex differences in the rate of endosteal apposition and resorption (Garn et al., 1972). Garn et al. (1972) found endosteal apposition began earlier and was in greater magnitude in girls than in boys as a result of the estrogen surge at puberty. The authors postulated that female endosteal bone is accrued during puberty in anticipation of future reproductive needs and to minimize bone loss secondary to diminished estrogen levels in later life.

As is the case in many species of vertebrates, there is a pronounced sex difference in bone mass in rats. Sexually mature males have longer, more robust long bones than comparably-aged females (Turner et al., 1994b). In this study, we have shown that histomorphometric examination of the proximal tibial metaphysis reveals a higher cancellous bone volume (normalized to tissue volume) in female rats compared to male. The more abundant cancellous bone mass in females can be attributed to estrogen. Estrogen secretion at puberty reduces resorption of calcified growth plate cartilage during endochondral ossification (Budy et al., 1952; Lindquist et al., 1960) by inhibiting the fusion of chondroclasts from their circulating precursors (Tunner et al., 1994b). The

volume of primary spongiosa is thus increased without an increase in longitudinal bone growth (Tunner et al., 1994a). Increased retention of primary spongiosa provides a more extensive template for deposition of bone matrix by differentiating osteoblasts, leading to an increase in cancellous bone volume without an accompanying increase in bone formation rate (perimeter referent) (Tunner et al., 1994a). The greater cancellous bone volume in female rats was associated with differences in bone architecture, and the higher bone formation rate was due to an increase in mineralizing surface. These bone characteristics due to sex steroids were also confirmed in our study, we found there were more cortical bone area in tibia shaft and thicker trabeculea in male rat pups, whereas higher intrinsic stiffness at the femur shaft in females at D21; there were more bone mineral content, larger DXA derived bone area, femur diameter and length in male rats but more endosteal mineral surface in females at D60 (Table 11). At D60, bone mineral content and bone area by DXA were greater in DMT male rats, whereas there were no differences between treatments in females (Figure 9).

Examination of bone formation characteristics in young adult female rats in the current study revealed increases in the mineralizing surface for animals that received DMT in early life. This finding is consistent with the decreased eroded surface, a bone resorption parameter, after adjusting for soft tissue lean mass in the same animals, indicating an increase in bone formation coupled with a decrease in bone turnover and resorption. The mineral apposition rate, a parameter reflecting the activity of osteoblasts, was also increased in the DMT female cohort. It appears that DMT suppressed bone resorption and increased bone formation resulting in a positive bone gain and a possible better connectivity of trabeculae. Taken together, the positive balance between bone

formation and resorption and the improvement of microarchitecture contributed to the efficacy of DMT on bone mass and structure. The positive findings observed in young adult female DMT rats require further study to confirm potential benefits of DMT during early postnatal life on lifelong bone structure, strength, and health.

There are 38 eta^2 values above the threshold of 0.06 for a "medium" effect (Cohen, 1988) out of all 73 nonsignificant effects of DMT on periosteal, endosteal and cancellous bone development and mineralization, bone density and strength (Tables 12 & 13). For example, DMT male rats had greater DXA derived bone area (p=0.061, eta^2=0.37, and statistical power = 48%), increased mineral surface on cancellous bone (p=0.056, eta^2=0.39, and statistical power = 50%) at D21, and had greater trabecular thickness than CTL at D60 (p=0.098, eta^2=0.30, and statistical power = 38%); DMT females had decreased eroded surface on cancellous bone surface (p=0.064, eta^2=0.41, and statistical power = 48%) than CTL at D21, and had more cortical area on the tibia shaft (p=0.061, eta^2=0.42, and statistical power = 49%), increased periosteal bone formation rate (p=0.093, eta^2=0.35, and statistical power = 39%), greater trabecular number on cancellous bone area (p=0.101, eta^2=0.34, and statistical power = 37%), decreased trabecular space (p=0.071, eta^2=0.39, and statistical power = 45%), increased mineral surface on cancellous bone surface (p=0.105, eta^2=0.33, and statistical power = 36%) and greater diameter on femur shaft (p=0.104, eta^2=0.33, and statistical power = 37%) compared to CTL at D60. Thus, these nonsignificant findings should be cautiously interpreted since the eta^2 values ranged from 0.30 to 0.41, yet the statistical power ranged from only 36% to 50%. Here again, these variables support the conclusion above that the females experienced a more sensitive and earlier impact of DMT on histomorphometric

variables in the juvenile period (D21), and then developed a prolonged, positive influence of DMT on bone strength at young age (D60).

In summary, treatment of newborn rat pups with DMT elicited an anabolic effect on postnatal growth and subsequent bone growth and development. DMT improved soft tissue lean mass deposition, stimulated bone mineral apposition on both the cortical and cancellous bone surfaces, tended to improve the microarchitecture and to decrease bone resorption on the trabecular bone surface of proximal tibia metaphysis. The current study opens a door to further study the presence of positive complementary interactions between DMT in early postnatal life and the GH/IGF-1 axis, sex steroids, and the muscle-bone relationship. The lack of biochemical markers as well as absence of body composition and bone studies in older animals (>60D) suggests a need for future studies to verify a prolonged, positive impact of early life DMT on skeletal health.

Limitations of the Study

Our primary interest was in delineating DMT treatment effects on bone growth, mineralization and strength, but there are methodological limitations to our measurements. The body composition is derived from DXA, a two-dimensional image, and we assumed the soft tissue lean mass incorporated all muscle attachments to bone that then exerted mechanical stimulus to bone formation. A better way would have been to dissect all the muscles from the skeleton and weigh them accurately. Secondly, there was no calculation for stress (σ=Force/Area, Pa) in the MTS system for femur shaft three-point bending. Such a measure indicates the magnitude of the force applied to a material of a defined cross-sectional area. Such controlled loading conditions aid in the

direct comparision of the properties of one material (e.g., cortical bone from a femur of an 80-year-old woman) to another (e.g., cortical bone from a 20-year-old woman), or to facilitate comparisons between laboratories. An apparent limitation of this study is the absence of serum and urinary markers of bone formation and resorption as well as neuroendocrine-related growth factors and stress hormones. Collection of these biomarkers might have provided mechanistic insights on how DMT during early postnatal life modulates stress, subsequent IGF1 activity, growth patterns, and adult body composition. In addition, the small sample size may have decreased the statistical power. Due to the limited sample size, the present study is insufficiently powered to detect the DMT effects on bone growth and mineralization. We could speculate whether if the sample size was larger and up to 10 or more per group, then more results of bone variables in DMT would be statistically different from CTL. This is particularly the case for the analyses with higher eta^2 values but slightly lower statistical power. Finally, it should be emphasized that our results only apply to a rat model with normal neonatal stress conditions. These results do not apply to any neonatal stress such as premature birth or mother-separation.

Implications for Future Research

For further understanding of the DMT effects on bone formation in this rat model, more sophisticated histomorphometric studies, such as the quantification of remodeling, minimodeling and mixed remodeling/minimodeling of formation sites, would help determine the relationship between DMT and these mechanisms and the contribution of this interaction on positive bone balance. These quantification techniques,

unfortunately, are tedious as they involve serial analysis of fluorochrome labeled cement line stained sections (Cui et al., 2001; Erben, 2001, 2003). Nevertheless, such an effort would help to better understand how potentially anabolic effects of DMT increase bone mass and strength. The compression test for lumbar vertebral bodies also would be needed for better estimation the DMT impacts on important skeletal sites. Further, stress would be tested in both the three-point bending and compression tests. The gastrocnemius, soleus and quadriceps muscles attached to the tibia would be stripped off and weighed and correlated with tibial shaft periosteal mineralization and bone formation, to precisely establish the muscle-bone relation. DMT intervention would be applied to the neonatal stress rat model such as premature birth or mother-separation, in order to clarify the anabolic effects of the DMT mechanism under clinical environments. It would also be helpful to assess biomarkers of bone formation (serum type I collagen C-terminal propeptide [PICP]) and resorption (urine pyridinoline cross-links of collagen [Pyd]); serum calcium, phosphate, alkaline phosphatase, parathyroid hormone (PTH), and 1,25-(OH)2 vitamin D; and urine levels of calcium, phosphate, and creatinine. Future research that included these measures would confirm the synergistic effects from DMT treatment on bone density, strength and histomorphometry. In addition, other serum hormones like sex steroids, GH/IGF, catecholatemine and glucoticoids levels would also be tested for illustrating the efficacy of DMT on neuroendocrine variables and its interaction with the potential anabolic impact on bone. Finally, studying older animals (mature adult and elderly) would provide additional insight into whether DMT in early postnatal life has the potential to enhance lifelong bone health and minimize age-related bone loss and its associated morbidities.

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